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(54) Title: A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS (57) Abstract This application describes a high throughput assay for screening for compounds which are capable of binding to a fusion protein which consists of a target protein and an FK506-binding protein.		

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TITLE OF THE INVENTION**A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS****BACKGROUND OF THE INVENTION**

5 Src homology 2 (SH2) domains are a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. They have routinely been expressed in *E. coli* as fusion proteins with glutathione-S-transferase (GST). This usually provides high level expression and
10 straightforward affinity purification on glutathione-Sepharose. Ligand binding is then assayed by incubating the GST/SH2 with a radiolabeled phosphopeptide, precipitating the complex with glutathione-Sepharose, washing the beads, and then counting the beads to determine bound radioactivity [Isakov et al., *J. Exp. Med.*, 181, 375-380 (1995); Piccione
15 et al., *Biochemistry*, 32, 3197-3202 (1993); Huyer et al., *Biochemistry*, 34, 1040-1049 (1995)]. There are several disadvantages to this procedure, particularly when applied to high-throughput screening for agonists, antagonists, or inhibitors as new leads for drug development. First, the radiolabeling of the peptide is carried out either enzymatically
20 with a kinase and [^{32}P]ATP or chemically with [^{125}I]Bolton-Hunter reagent. In both cases, the isotopes are short-lived and thus require frequent preparation of material. In the case of enzymatic phosphorylation, the appropriate kinase must also be available in sufficient quantity to generate enough material for screening purposes.
25 Second, the protocol requires separation of bound complex from free phosphopeptide by washing of the glutathione-Sepharose beads. This is a nonequilibrium procedure that risks dissociation of the bound ligand, particularly when off-rates are fast. Thus, there is the possibility of misleading results. Finally, due to the number of manipulations and
30 centrifugations involved, the protocol is very tedious to conduct manually and is not readily adaptable to robotic automation to increase throughput.

Two additional methods for measuring the interaction of proteins and ligands that have been applied to SH2 domains are biospecific interaction analysis using surface plasmon resonance and

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isothermal titration calorimetry (Felder et al., *Mol. Cell. Biol.*, 13, 1449-1455 (1993); Panayotou et al., *Mol. Cell. Biol.*, 13, 3567-3576 (1993); Payne et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 4902-4906 (1993); Morelock et al., *J. Med. Chem.* 38, 1309-18 (1995); Ladbury et al., *Proc. Natl. Acad. Sci. U.S.A.*, 92, 3199-3203 (1995); Lemmon et al., *Biochemistry*, 33, 5070-5076 (1994)). These techniques do not require a particular fusion partner for the SH2 domain, but do require sophisticated instrumentation that is not amenable to high throughput screening.

10 **SUMMARY OF THE INVENTION**

The instant invention covers a method of screening for compounds capable of binding to a fusion protein which comprises combining a test compound, a tagged ligand, a fusion protein (target protein, peptide linker and FK506-binding protein), a radiolabeled ligand, and coated scintillation proximity assay (SPA) beads, and then measuring the scintillation counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on the binding of the tagged ligand. This invention provides an immediate means of making use of SPA technology for the functional assay of ligand binding to a single or multiple signal transduction domain(s), for example a phosphopeptide binding to an SH2 domain. The present invention does not require specialized radiochemical synthesis and is readily adaptable to robotic automation for high capacity screening for agonists, antagonists, and/or inhibitors.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

- A.) Binding of the streptavidin SPA bead, biotinylated ligand and the fusion protein (SH2:FKBP), which emits a detectable signal; and
- 5 B.) Binding of the test compound and the fusion protein (SH2:FKBP), which results in no signal detection .

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention relates to a method of screening for compounds which preferentially bind to a target protein.

An embodiment of this invention is a method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

- 15 a) mixing a test compound, a tagged ligand, the fusion protein, a radiolabeled ligand and coated scintillation proximity assay (SPA) beads;
- b) incubating the mixture for between about 1 hour and about 24 hours;
- 20 c) measuring the SPA bead-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and
- 25 d) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

The term "fusion protein" refers to a "target protein" fused to an "FK506-binding protein" (FKBP), the two proteins being separated by a "peptide linker".

- 30 A "peptide linker" may consist of a sequence containing from about 1 to about 20 amino acids, which may or may not include the sequence for a protease cleavage site. An example of a peptide linker which is a protease cleavage site is represented by the amino acid sequence GLPRGS.

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The term "target protein" refers to any protein that has a defined ligand. Included within this definition of target protein are single and multiple signal transduction domains, such as, but not limited to, Src homology 1 (SH1), Src homology 2 (SH2), Src homology 3 (SH3), and pleckstrin homology (PH) domains [Hanks & Hunter, *FASEB J.*, 9, 576-596 (1995); Bolen, *Curr. Opin. Immunol.*, 7, 306-311 (1995); Kuriyan & Cowburn, *Curr. Opin. Struct. Biol.*, 3, 828-837 (1993); Cohen et al., *Cell*, 80, 237-248 (1995)]. The term "SH1 domain" refers to a family of homologous protein domains that bind ATP and catalyze tyrosine phosphorylation of peptide and protein substrates. The term "SH2 domain" refers to a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. The term "SH3 domain" refers to a family of homologous protein domains that share the common property of recognizing polyproline type II helices. The term "PH domain" refers to a family of homologous protein domains that mediate both protein-protein and protein-lipid interactions. Examples of SH2 domains which may be utilized in the method of the invention include, but are not limited to, the single and tandem SH2 domains present in the tyrosine kinases ZAP, SYK and LCK. The DNA sequences were obtained from GenBank, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894. The Accession Numbers for the sequences are: human ZAP (L05148); human SYK (L28824) and human LCK (X13529).

The term "tagged ligand" refers to a biotinylated or epitope tagged ligand for the target protein.

The term "radiolabeled ligand" refers to a [^3H]- or [^{125}I]-labeled ligand which binds to the FKBP. An example of a radiolabeled ligand useful in the instant invention is [^3H]-dihydroFK506.

The term "coated scintillation proximity assay beads" (SPA beads) refers to streptavidin-coated scintillation proximity assay beads when the tagged ligand is biotinylated, and to anti-epitope antibody bound to anti-antibody-coated or protein A-coated scintillation proximity assay beads when the tagged ligand is epitope-tagged.

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The term "control assay" refers to the assay when performed in the presence of the tagged ligand, the fusion protein, the radiolabeled ligand and the coated scintillation proximity assay beads, but in the absence of the test compound.

- 5 The term FK506-binding proteins may include, but are not limited to, the below listed FKBP and FKBP homologues, which include a citation to the references which disclose them. This list is not intended to limit the scope of the invention.

10 **Mammalian**

FKBP-12

Galat et al., *Eur. J. Biochem.*, 216:689-707 (1993).

FKBP-12.6

Wiederrecht, G. and F. Etzkorn
Perspectives in Drug Discovery and Design, 2:57-84 (1994).

15

FKBP-13

Galat et al., *supra*; Wiederrecht and Etzkorn, *supra*.

FKBP-25

Galat et al., *supra*; Wiederrecht and Etzkorn, *supra*.

20

FKBP-38

Wiederrecht and Etzkorn, *supra*.

FKBP-51

Baughman et al., *Mol. Cell. Biol.*, 8, 4395-4402(1995).

FKBP-52

Galat et al., *supra*.

25 **Bacteria**

Legionella pneumophila

Galat et al., *supra*.

Legionella micadei

Galat et al., *supra*.

Chlamydia trachomatis

Galat et al., *supra*.

E. coli fkpa

Horne, S.M. and K.D. Young, *Arch. Microbiol.*, 163:357-365 (1995).

30

E. coli slyD

Roof et al., *J. Biol. Chem.* 269:2902-2910 (1994).

E. coli orf149

Trandinh et al., *FASEB J.* 6:3410-3420 (1992).

- 6 -

Neisseria meningitidis Hacker, J. and G. Fischer, *Mol. Micro.*,
10:445-456 (1993).
Streptomyces chrysomallus Hacker and Fischer, *supra*.

5 **Fungal**

yeast FKBP-12 Cardenas et al., *Perspectives in Drug
Discovery and Design* , 2:103-126
(1994).
yeast FKBP-13 Cardenas et al., *supra*.
10 yeast NPR1(FPR3) Cardenas et al., *supra*.
Neurospora Galat et al., *supra*.

 A variety of host cells may be used in this invention,
which include, but are not limited to, bacteria, yeast, bluegreen algae,
15 plant cells, insect cells and animal cells.

 Expression vectors are defined herein as DNA sequences
that are required for the transcription of cloned copies of genes and the
translation of their mRNAs in an appropriate host. Such vectors can
be used to express genes in a variety of host cells, such as, bacteria,
20 yeast, bluegreen algae, plant cells, insect cells and animal cells.

 Specifically designed vectors allow the shuttling of DNA
between hosts such as bacteria-yeast or bacteria-animal cells. An
appropriately constructed expression vector may contain: an origin of
replication for autonomous replication in host cells, selectable markers, a
25 limited number of useful restriction enzyme sites, a potential for high
copy number, and active promoters. A promoter is defined as a DNA
sequence that directs RNA polymerase to bind to DNA and initiate RNA
synthesis. A strong promoter is one which causes mRNAs to be initiated
at high frequency. Expression vectors may include, but are not limited
30 to, cloning vectors, modified cloning vectors, specifically designed
plasmids or viruses. Commercially available vectors suitable for FKBP
fusion protein expression include, but are not limited to pBR322
(Promega), pGEX (Amersham), pT7 (USB), pET (Novagen), pIBI (IBI),
pProEX-1 (Gibco/BRL), pBluescript II (Stratagene), pTZ18R and

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pTZ19R (USB), pSE420 (Invitrogen), pVL1392 (Invitrogen), pBlueBac (Invitrogen), pBacPAK (Clontech), pHIL (Invitrogen), pYES2 (Invitrogen), pCDNA (Invitrogen), pREP (Invitrogen) or the like.

5 The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation.

10 *E. coli* containing an expression plasmid with the target gene fused to FKBP are grown and appropriately induced. The cells are then pelleted and resuspended in a suitable buffer. Although FKBP-12 lacks sequences that specifically direct it to the periplasm, FKBP fusions are primarily located there and can be released by a standard freeze/thaw treatment of the cell pellet. Following centrifugation, the resulting supernatant contains >80% pure FKBP fusion, which if desired can be
15 purified further by conventional methods. Alternatively, the assay is not dependent on pure protein and the initial periplasmic preparation may be used directly. A thrombin site located between FKBP and the target protein can be used as a means to cleave FKBP from the fusion; such cleaved material may be a suitable negative control for subsequent
20 assays.

A fusion protein which contains a single or multiple SH2 domain(s) may be purified by preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support. A freeze/thaw extract is prepared from
25 the cells which express the fusion protein and is loaded onto the affinity matrix. The desired fusion protein is then specifically eluted with phenyl phosphate.

To assay the formation of a complex between a target protein and its ligand, the tagged ligand is mixed with the FKBP fusion protein in a suitable buffer in the presence of the radiolabeled ligand in
30 the well of a white microplate. After a suitable incubation period to allow complex formation to occur, coated SPA beads are added to capture the tagged ligand and any bound fusion protein. The plate is sealed, incubated for a sufficient period to allow the capture to go to

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completion, then counted in a multiwell scintillation counter. Screening for agonists/antagonists/inhibitors is carried out by performing the initial incubation prior to the capture step with SPA beads in the presence of a test compound(s) to determine whether they have an effect upon the binding of the tagged ligand to the fusion protein. This principle is illustrated by Figure 1.

The present invention can be understood further by the following examples, which do not constitute a limitation of the invention.

EXAMPLE 1

Process for Preparing the FKBP fusion cloning vector

General techniques for modifying and expressing genes in various host cells can be found in Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology (John Wiley & Sons, New York, New York, 1989). Sequences for a 3'- altered FKBP fragment that contained a glycine codon (GGT) in place of the stop (TGA) codon followed by a sequence encoding a thrombin site (Leu-Val-Pro-Arg) and *Bam*HI restriction site (GAATTC) were amplified using the polymerase chain reaction (PCR). The PCR reaction contained the following primers: 5'-GATCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCA-3' and 5'-TACGAATTCTGGCGTGGATCCACGCGGAACCAGACCTTCCAGT TTTAG-3' and a plasmid containing human FKBP-12 as the template. The resulting 367 base pair amplification product was ligated into the vector pCRII (Invitrogen) and the ligation mixture transformed into competent *Escherichia coli* cells. Clones containing an insert were identified using PCR with flanking vector primers. Dideoxy DNA sequencing confirmed the nucleotide sequence of one positive isolate. The altered 338 base pair FKBP fragment was excised from the pCRII plasmid using *Nco*I and *Bam*HI and ligated into *Nco*I and *Bam*HI digested pET9d (Novagen) plasmid. Competent *E. coli* were transformed with the ligation mixture, and colonies containing the insert were identified using PCR with primers encoding for flanking vector sequences. The FKBP fusion cloning vector is called pET9dFKBPt.

EXAMPLE 2

Process for Preparing the FK-ZAP fusion expression vector

5 A DNA fragment encoding for the tandem SH2 domains of ZAP-70 was prepared by PCR to contain a *Bam*HI site at the 5'-end such that the reading frame was conserved with that of FKBP in the fusion vector. At the 3'-end, the fragment also incorporated a stop codon followed by a *Bam*HI site. The PCR reaction contained Molt-4 cDNA
10 (Clontech) and the following primers:
5'-ATTAGGATCCATGCCAGATCCTGCAGCTCACCTGCCCT-3' and
5'-ATATGGATCCTTACCAGAGGCGTTGCT-3'. The fragment was cloned into a suitable vector, sequenced, digested with *Bam*HI, and the insert containing the SH2 domains ligated to *Bam*HI treated
15 pET9dFKBPt, and transformed into *E. coli*. Clones containing inserts in the correct orientation were identified by PCR or restriction enzyme analysis. Plasmid DNA was prepared and used to transform BL21(DE3) cells.

20

EXAMPLE 3

Process for Preparing the FK-SYK fusion expression vector

 The expression vector for the tandem SH2 domains of Syk fused to FKBP was prepared as in Example 2 except that the PCR
25 reaction contained Raji cell cDNA (Clontech) and the following primers:
5'-CAATAGGATCCATGGCCAGCAGCGGCATGGCTGA-3' and
5'-GACCTAGGATCCCTAATTAACATTTCCCTGTGTGCCGAT-3'.

30

EXAMPLE 4

Process for Preparing the FK-LCK fusion expression vector

 The expression vector for the SH2 domain of Lck fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:

- 10 -

5'-ATATGGATCCATGGCGAACAGCCTGGAGCCCGAACCCT-3'
and
5'-ATTAGGATCCTTAGGTCTGGCAGGGGCGGCTCAACCGTGT
GCA-3'.

5

EXAMPLE 5

FK-ZAP

10 Step A: Process for Expression of FK-ZAP

E. coli BL21(DE3) cells containing the pET9dFKBPt/
ZapSH2 plasmid were grown in Luria-Bertani (LB) media containing 50
microgram/ml kanamycin at 37 degrees C until the optical density
measured at 600 nm was 0.5-1.0. Expression of the FK-ZAP fusion
15 protein was induced with 0.1 mM isopropyl beta-thiogalactopyranoside
and the cells were grown for another 3-5 hr at 30 degrees C. They were
pelleted at 4400 x g for 10 min at 4 degrees C and resuspended in 2% of
the original culture volume with 100 mM tris pH 8.0 containing 1
microgram/ml each aprotinin, pepstatin, leupeptin, and bestatin. The
20 resuspended pellet was frozen at -20 degrees C until further purification.

Step B: Process for Purification of FK-ZAP

The affinity matrix for purification of FK-ZAP was prepared
by combining agarose-immobilized avidin with excess biotinylated
25 phosphopeptide derived from the ζ 1 ITAM sequence of the human T-cell
receptor, biotinyl-GSNQLpYNELNLGRREEpYDVLDK, and washing
out unbound peptide. Frozen cells containing FK-ZAP were thawed in
warm water, refrozen on dry ice for about 25 min., then thawed again.
After the addition of 0.1% octyl glucoside, 1 mM dithiothreitol (DTT)
30 and 500 mM NaCl, the extract was centrifuged at 35,000 x g for
approximately 30 minutes. The supernatant was loaded onto the
phosphopeptide affinity column, at about 4° and washed with phosphate
buffered saline containing 1 mM DTT and 0.1% octyl glucoside.
FK-ZAP was eluted with 200 mM phenyl phosphate in the same buffer at
35 about 37°. The protein pool was concentrated and the phenyl phosphate

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removed on a desalting column. The purified FK-ZAP was stored at about -30° in 10 mM HEPES/150 mM NaCl/1 mM DTT/0.1 mM EDTA/10% glycerol.

5

EXAMPLE 6

FK-SYK

E. coli BL21(DE3) cells containing the pET9dFKBPt/SykSH2 plasmid were grown, induced, and harvested as described in Example 5. FK-SYK was purified using the same affinity matrix and methodology described in Example 5.

EXAMPLE 7

15 FK-LCK

E. coli BL21(DE3) cells containing the pET9dFKBPt/LckSH2 plasmid were grown, induced, and harvested as described in Example 5. The affinity matrix for purification of FK-LCK was prepared by combining agarose-immobilized avidin with excess biotinyl-EPQpYEEIPIYL, and washing out unbound peptide. The remaining methodology for purification was the same as Example 5.

EXAMPLE 8

25

Method of Screening for Antagonists of FK-ZAP

Assays were conducted at ambient temperature in a buffer consisting of 25 mM HEPES, 10 mM DTT, 0.01% TWEEN-20, pH 7.0. 10 µl of a DMSO solution of test compound(s) and 120 µl of biotinyl-phosphopeptide stock solution were dispensed into the wells of a 96-well Packard Optiplate. Next, 20 µl of a mixture of FK-ZAP protein and ³H-dihydroFK506 were added to each test well. Finally, 50 µl of a 4 mg/ml suspension of SPA beads were dispensed to each well. Final concentrations of the assay components were:

30

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25 nM biotinyl-GSNQLpYNELNLGRREEpYDVLDK
25 nM FK-ZAP fusion protein
10 nM ³H-dihydroFK506 (DuPont NEN)
1.0 mg/ml streptavidin-SPA beads (Amersham)
5% DMSO

5

The plate was sealed and incubated between 1 and 8 hours. Bead-bound radioactivity was then measured in a Packard Topcount microplate scintillation counter.

10

EXAMPLE 9

Method of Screening for Antagonists of FK-SYK

The assays were conducted as set forth in Example 8, except that FK-SYK replaced FK-ZAP.

15

EXAMPLE 10

Method of Screening for Antagonists of FK-LCK

The assays were conducted as set forth in Example 8, except that FK-LCK replaced FK-ZAP and the tagged ligand was 25 nM biotinyl-EPQpYEEIPIYL.

20

- 13 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Salowe, Scott P.
- (ii) TITLE OF INVENTION: A High Throughput Assay Using Fusion Proteins
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
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 - (C) CITY: Rahway
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 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
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 - (B) FILING DATE:
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- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Camara, Valerie J.
 - (B) REGISTRATION NUMBER: 35,090
 - (C) REFERENCE/DOCKET NUMBER: 19494
- (ix) TELECOMMUNICATION INFORMATION:
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 - (B) TELEFAX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1137 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

- 14 -

ATGGGAGTGC AGGTGGAAC CATCTCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC	60
CAGACCTGCG TGGTGCCTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC	120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG	180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT	240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCAC CACATGCCAC TCTCGTCTTC	300
GATGTGGAGC TTCTAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGCC AGATCCTGCA	360
GCTCACCTGC CCTTCTTCTA CGGCAGCATC TCGCGTGCCG AGGCCGAGGA GCACCTGAAG	420
CTGGCGGGCA TGGCGGACGG GCTCTTCCTG CTGCGCCAGT GCCTGCGCTC GCTGGGCGGC	480
TATGTGCTGT CGCTCGTGCA CGATGTGCGC TTCCACCACT TTCCCATCGA GCGCCAGCTC	540
AACGGCACCT ACGCCATTGC CGGCGGCAA GCGCACTGTG GACCGGCAGA GCTCTGCGAG	600
TTCTACTCGC GCGACCCCGA CGGGCTGCCC TGCAACCTGC GCAAGCCGTG CAACCGGCCG	660
TCGGGCCTCG AGCCGAGCC GGGGTCTTC GACTGCCTGC GAGACGCCAT GGTGCGTGAC	720
TACGTGCGCC AGACGTGGAA GCTGGAGGGC GAGGCCCTGG AGCAGGCCAT CATCAGCCAG	780
GCCCCGAGG TGGAGAAGCT CATTGCTACG ACGGCCACG AGCGGATGCC CTGGTACCAC	840
AGCAGCCTGA CGCGTGAGGA GGCCGAGCGT AAACCTTACT CTGGGGCGCA GACCGACGGC	900
AAGTTCCTGC TGAGGCCCGG GAAGGAGCAG GGCACATACG CCCTGTCCCT CATCTATGGG	960
AAGACGGTGT ACCACTACCT CATCAGCCAA GACAAGCGG GCAAGTACTG CATTCCCGAG	1020
GGCACCAAGT TTGACACGCT CTGGCAGCTG GTGGAGTATC TGAAGCTGAA GGCGGACGGG	1080
CTCATCTACT GCCTGAAGGA GGCCTGCCCC AACAGCAGTG CCAGCAACGC CTCTTAA	1137

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1155 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGGAGTGC AGGTGGAAC CATCTCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC	60
CAGACCTGCG TGGTGCCTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC	120

- 15 -

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CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG      180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT      240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC      300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC CAGCAGCGGC      360
ATGGCTGACA GCGCCAACCA CCTGCCCTTC TTTTTCGGCA ACATCACCCG GGAGGAGGCA      420
GAAGATTACC TGGTCCAGGG GGCATGAGT GATGGGCTTT ATTTGCTGCG CCAGAGCCGC      480
AACTACCTGG GTGGCTTCGC CCTGTCCGTG GCCCACGGGA GGAAGGCACA CCACTACACC      540
ATCGAGCGGG AGCTGAATGG CACCTACGCC ATCGCCGGTG GCAGGACCCA TGCCAGCCCC      600
GCCGACCTCT GCCACTACCA CTCCCAGGAG TCTGATGGCC TGGTCTGCCT CCTCAAGAAG      660
CCCTTCAACC GGCCCCAAGG GGTGCAGCCC AAGACTGGGC CCTTTGAGGA TTTGAAGGAA      720
AACCTCATCA GGAATATGT GAAGCAGACA TGGAACCTGC AGGGTCAGGC TCTGGAGCAG      780
GCCATCATCA GTCAGAAGCC TCAGCTGGAG AAGCTGATCG CTACCACAGC CCATGAAAAA      840
ATGCCTTGGT TCCATGGAAA AATCTCTCGG GAAGAATCTG AGCAAATTGT CCTGATAGGA      900
TCAAAGACAA ATGGAAAGTT CCTGATCCGA GCCAGAGACA ACAACGGCTC CTACGCCCTG      960
TGCCTGCTGC ACGAAGGGAA GGTGCTGCAC TATCGCATCG ACAAAGACAA GACAGGGAAG     1020
CTCTCCATCC CCGAGGGAAA GAAGTTCGAC ACGCTCTGGC AGCTAGTCGA GCATTATTCT     1080
TATAAAGCAG ATGGTTTGTT AAGAGTTCTT ACTGTCCCAT GTCAAAAAAT CGGCACACAG     1140
GGAATGTTA ATTAG                                     1155

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 675 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

ATGGGAGTGC AGGTGGAAAC CATCTCCCA GAGATGGAC GCACCTTCCC CAAGCGCGGC      60
CAGACCTGCG TGGTGCAC TA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCTCTC     120

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CCGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
 GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
 TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCAC CACATGCCAC TCTCGTCTTC 300
 GATGTGAGC TTCTAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC GAACAGCCTG 360
 GAGCCCGAAC CCTGGTTCTT CAAGAACCTG AGCCGCAAGG ACGCGGAGCG GCAGCTCCTG 420
 GCGCCCGGA AACTCACGG CTCCTTCCTC ATCCGGGAGA GCGAGAGCAC CGCGGGATCG 480
 TTTTCACTGT CGGTCCGGGA CTTCGACCAG AACCAGGGAG AGGTGGTGAA ACATTACAAG 540
 ATCCGTAATC TGGACAACGG TGGCTTCTAC ATCTCCCCTC GAATCACTTT TCCCGGCCCTG 600
 CATGAACTGG TCCGCCATTA CACCAATGCT TCAGATGGGC TGTGCACACG GTTGAGCCGC 660
 CCCTGCCAGA CCTAA 675

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 378 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1 5 10 15
 Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
 20 25 30
 Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
 35 40 45
 Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
 50 55 60
 Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
 65 70 75 80
 Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
 85 90 95
 Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
 100 105 110

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Arg Gly Ser Met Pro Asp Pro Ala Ala His Leu Pro Phe Phe Tyr Gly
 115 120 125
 Ser Ile Ser Arg Ala Glu Ala Glu Glu His Leu Lys Leu Ala Gly Met
 130 135 140
 Ala Asp Gly Leu Phe Leu Leu Arg Gln Cys Leu Arg Ser Leu Gly Gly
 145 150 155 160
 Tyr Val Leu Ser Leu Val His Asp Val Arg Phe His His Phe Pro Ile
 165 170 175
 Glu Arg Gln Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Lys Ala His
 180 185 190
 Cys Gly Pro Ala Glu Leu Cys Glu Phe Tyr Ser Arg Asp Pro Asp Gly
 195 200 205
 Leu Pro Cys Asn Leu Arg Lys Pro Cys Asn Arg Pro Ser Gly Leu Glu
 210 215 220
 Pro Gln Pro Gly Val Phe Asp Cys Leu Arg Asp Ala Met Val Arg Asp
 225 230 235 240
 Tyr Val Arg Gln Thr Trp Lys Leu Glu Gly Glu Ala Leu Glu Gln Ala
 245 250 255
 Ile Ile Ser Gln Ala Pro Gln Val Glu Lys Leu Ile Ala Thr Thr Ala
 260 265 270
 His Glu Arg Met Pro Trp Tyr His Ser Ser Leu Thr Arg Glu Glu Ala
 275 280 285
 Glu Arg Lys Leu Tyr Ser Gly Ala Gln Thr Asp Gly Lys Phe Leu Leu
 290 295 300
 Arg Pro Arg Lys Glu Gln Gly Thr Tyr Ala Leu Ser Leu Ile Tyr Gly
 305 310 315 320
 Lys Thr Val Tyr His Tyr Leu Ile Ser Gln Asp Lys Ala Gly Lys Tyr
 325 330 335
 Cys Ile Pro Glu Gly Thr Lys Phe Asp Thr Leu Trp Gln Leu Val Glu
 340 345 350
 Tyr Leu Lys Leu Lys Ala Asp Gly Leu Ile Tyr Cys Leu Lys Glu Ala
 355 360 365
 Cys Pro Asn Ser Ser Ala Ser Asn Ala Ser
 370 375

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
 (B) TYPE: amino acid

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(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1           5           10           15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
 20           25           30
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
 35           40           45
Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
 50           55           60
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
 65           70           75           80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
 85           90           95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
 100          105          110
Arg Gly Ser Met Ala Ser Ser Gly Met Ala Asp Ser Ala Asn His Leu
 115          120          125
Pro Phe Phe Phe Gly Asn Ile Thr Arg Glu Glu Ala Glu Asp Tyr Leu
 130          135          140
Val Gln Gly Gly Met Ser Asp Gly Leu Tyr Leu Leu Arg Gln Ser Arg
 145          150          155          160
Asn Tyr Leu Gly Gly Phe Ala Leu Ser Val Ala His Gly Arg Lys Ala
 165          170          175
His His Tyr Thr Ile Glu Arg Glu Leu Asn Gly Thr Tyr Ala Ile Ala
 180          185          190
Gly Gly Arg Thr His Ala Ser Pro Ala Asp Leu Cys His Tyr His Ser
 195          200          205
Gln Glu Ser Asp Gly Leu Val Cys Leu Leu Lys Lys Pro Phe Asn Arg
 210          215          220
Pro Gln Gly Val Gln Pro Lys Thr Gly Pro Phe Glu Asp Leu Lys Glu
 225          230          235          240

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Asn Leu Ile Arg Glu Tyr Val Lys Gln Thr Trp Asn Leu Gln Gly Gln
 245 250 255
 Ala Leu Glu Gln Ala Ile Ile Ser Gln Lys Pro Gln Leu Glu Lys Leu
 260 265 270
 Ile Ala Thr Thr Ala His Glu Lys Met Pro Trp Phe His Gly Lys Ile
 275 280 285
 Ser Arg Glu Glu Ser Glu Gln Ile Val Leu Ile Gly Ser Lys Thr Asn
 290 295 300
 Gly Lys Phe Leu Ile Arg Ala Arg Asp Asn Asn Gly Ser Tyr Ala Leu
 305 310 315 320
 Cys Leu Leu His Glu Gly Lys Val Leu His Tyr Arg Ile Asp Lys Asp
 325 330 335
 Lys Thr Gly Lys Leu Ser Ile Pro Glu Gly Lys Lys Phe Asp Thr Leu
 340 345 350
 Trp Gln Leu Val Glu His Tyr Ser Tyr Lys Ala Asp Gly Leu Leu Arg
 355 360 365
 Val Leu Thr Val Pro Cys Gln Lys Ile Gly Thr Gln Gly Asn Val Asn
 370 375 380

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1 5 10 15
 Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
 20 25 30
 Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
 35 40 45
 Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
 50 55 60

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Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65 70 75 80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
85 90 95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
100 105 110
Arg Gly Ser Met Ala Asn Ser Leu Glu Pro Glu Pro Trp Phe Phe Lys
115 120 125
Asn Leu Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro Gly Asn
130 135 140
Thr His Gly Ser Phe Leu Ile Arg Glu Ser Glu Ser Thr Ala Gly Ser
145 150 155 160
Phe Ser Leu Ser Val Arg Asp Phe Asp Gln Asn Gln Gly Glu Val Val
165 170 175
Lys His Tyr Lys Ile Arg Asn Leu Asp Asn Gly Gly Phe Tyr Ile Ser
180 185 190
Pro Arg Ile Thr Phe Pro Gly Leu His Glu Leu Val Arg His Tyr Thr
195 200 205
Asn Ala Ser Asp Gly Leu Cys Thr Arg Leu Ser Arg Pro Cys Gln Thr
210 215 220

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WHAT IS CLAIMED IS:

1. A method of screening for compounds capable of binding to a fusion protein which comprises the steps of:
 - 5 a) mixing a test compound, a tagged ligand, the fusion protein, a radiolabeled ligand and coated scintillation proximity assay (SPA) beads;
 - b) incubating the mixture from between about 1 hour to about 24 hours;
 - 10 c) measuring the SPA bead-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and
 - 15 d) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.
2. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 1, wherein the tagged
20 ligand is a biotinylated ligand or epitope-tagged ligand.
3. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 2, wherein scintillation proximity assay beads are streptavidin-coated or anti-antibody or protein
25 A-coated.
4. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 3, wherein the radiolabeled ligand consists of [³H]- or [¹²⁵I]-labeled FK506 analog.
30
5. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 4, wherein the fusion protein comprises an FK506-binding protein linked through a peptide linker to a target protein.

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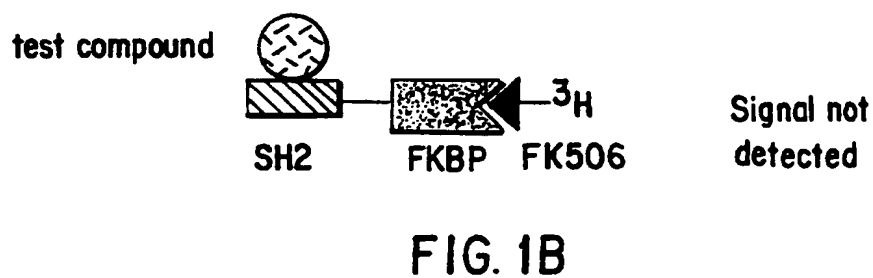
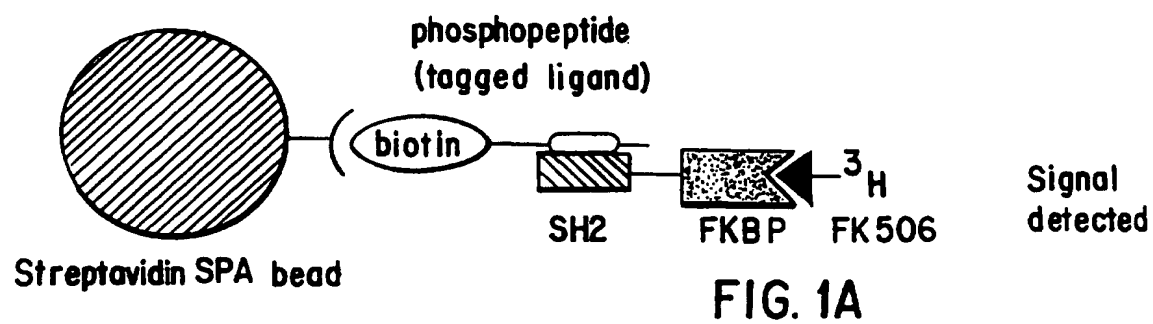
- 5 6. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 5, wherein the target protein comprises a single or multiple signal transduction domain.
- 10 7. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 6, wherein the single or multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.
- 15 8. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 7, wherein the target protein is a single or multiple SH2 domain.
- 20 9. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 8, wherein the radiolabeled ligand is [³H]-dihydroFK506.
- 25 10. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 9, wherein the FK506-binding protein is a 12kDA human FK506-binding protein.
- 30 11. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 10, wherein the target protein is a single or multiple SH2 domain selected from the group consisting of: ZAP:SH2, SYK:SH2 and LCK:SH2.
12. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, ZAP:SH2.
13. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, SYK:SH2.

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14. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, LCK:SH2.

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INTERNATIONAL SEARCH REPORT

Int. l. application No.
PCT/US96/14563

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/534

US CL : 435/7.5; 436/529

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.5; 436/529

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

search terms: scintillation proximity assay#, spa, fusion protein#, sh2 domain#, transduction domain#, target protein#, biotin?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sonatore et al. The Utility of FK506-Binding Protein as a Fusion Partner in Scintillation Proximity Assays: Application to SH2 Domains. Anal. Biochem. 1996, Vol. 240, pages 289-297.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to undermine the principle or theory underlying the invention
"E"	earlier documents published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"A"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 DECEMBER 1996

Date of mailing of the international search report

10 JAN 1997

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INTERNATIONAL SEARCH REPORT

Int. l. application No.
PCT/US96/14563

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lerner et al. Scintillation Proximity Assay for Human DNA Topoisomerase I using Recombinant Biotiny Fusion Protein Produced in Baculovirus-Infected Insect Cells. Anal. Biochem. 1996, Vol. 240, pages 289-297.	
Y	Skinner et al. Direct Measurement of the Binding of RAS to Neurobromin using a Scintillation Proximity Assay. Anal. Biochem. 1994, Vol. 223, pages 259-265.	1,3